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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV440868413 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Drew M.	Pardoll	Brookeville, MD

Additional inventors are being named on the one *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Immune Modulation through Targeting of the MINOR Gene

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☑ Specification *Number of Pages* 22 ☐ CD(s), Number _____

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☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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REGISTRATION NO. 45,282 *(if appropriate)*

Docket Number: 4406

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
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U.S. Provisional Patent Application

JHU Ref. No.: JHU-4406

Immune Modulation through targeting of the MINOR Gene

Inventors: Drew Pardoll, Katharine A. Whartenby, Kevin S. Gorski and Camie Chan

Johns Hopkins University School of Medicine
Office of Technology Licensing

Report of Invention Disclosure Form

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INVENTION INFORMATION

Title of Invention:
Immune Modulation through Targeting of the MINOR gene

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LTD Internal Use Only: JHU Ref.: 4406 TLA _____ Field of Use _____

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INVENTION DESCRIPTION

Describe the invention completely, using the outline given below.

1. Abstract of the Invention [Briefly describe the invention]

A number of vaccination strategies utilize dendritic cells (DCs) to immunize. While DCs are potent initiators of immune responses, their utility as vaccines may be limited by their relatively short *in vivo* lifespans. We have discovered a new gene in DCs that regulates DC apoptosis and have developed a strategy to inhibit expression of this gene, and by doing so, have shown that we can significantly enhance immune responses. This gene, termed , MINOR, for Mitogen Induced Nuclear Orphan Receptor,is a member of the Nur77 family of apoptosis-inducing genes, and its expression is highly and selectively upregulated in mature DCs, and, our data suggest that it plays a role in natural DC apoptosis. In order to enhance DC survival and function, we have developed a novel approach of inhibiting DC apoptosis via small interfering RNA **(siRNA)** technology. Our data suggest that inhibition of this gene leads to improvement of *ex vivo* DC vaccines and also utilize our system of bone marrow transplantation **(BMT)** with gene modified hematopoietic stem cells **(HSCs)** to analyze its effects on *de novo* generation of DCs *in vivo*.

2. Problem Solved [Describe the problem solved by this invention]

Dendritic cell vaccines have been developed for therapeutic use by generating the DCs both in vivo and in vitro, through various methods. However, these strategies have not been highly effective. Improving vaccination strategies for tumors is a significant goal of immunotherapy. It now appears that DC vaccines can elicit strong immune responses, but they are limited, in part by their short lifespans *in vivo*. While much emphasis has been placed in studying antigen **(Ag)** uptake, processing and presentation as well as costimulatory signal delivery by DCs, little is known about regulation of DC lifespan. Through investigating the unique pattern of gene expression, in DCs, we have identified one whose expression may be at least partly responsible for limiting the efficacy of DC vaccines due to its observed apoptosis-inducing effects. We show that by inhibiting this gene that we can prolong survival of DCs and also enhance immune responses. Thus, this is a novel approach to improving DC vaccines. **(Claims on attached page)**

3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art]

This report describes a new gene that is important for DC function. Expression of the new gene, MINOR, regulates apoptosis in DCs and is likely to be a limiting factor in immunogenicity of these cells. Inhibition of this gene provides a novel means to prolong the immune response in a number of settings. Potentiation of gene expression may also provide a means to inhibit the immune response in order to target hyper or autoimmune type processes.

4. Detailed Description of the invention:

On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

1- data pertaining to the invention;
2- drawings or photographs illustrating the invention;
3- structural formulae if a chemical;
4- procedural steps if a process
5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.

5. Workable Extent/Scope [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a ***compound,*** describe substitutions, breadth of substituents, derivatives, salts etc., if ***DNA or other biological material***, describe modifications that are expected to be operable, if a ***machine or device***, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

Future plans will center on applying inhibition of this gene in order to enhance immunogenicity in a number of different types of vaccine therapies. In addition, investigation into immunosuppression by potentiating expression of this gene is another therapeutic goal.

MINOR
Sequence

Katie Whartenby, Ph.D.
Assistant Professor of Oncology

atgccctgcgtgcaagcccagtatagcccttcacctccggggtccactta
cgccacgcagacttatggctcggaatacaccacagaaatcatgaaccccg
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BACKGROUND AND SIGNIFICANCE:

Improving vaccination strategies for tumors is a significant goal of immunotherapy. As a result of the potency of dendritic cells (DCs) as antigen presenting cells (APCs), DCs have been investigated for both their biology and potential as therapeutic agents. While many advances have occurred in this field, highly potent and durable anti-tumor immune responses have been difficult to achieve through these vaccines. It now appears that DC vaccines can elicit strong immune responses, but they are limited, in part by their short lifespans *in vivo*. While much emphasis has been placed in studying antigen (Ag) uptake, processing and presentation as well as costimulatory signal delivery by DCs, little is known about regulation of DC lifespan. Emerging technology has allowed the identification of genes that may be important to DC function, based on their relative expression levels. Since DCs have a unique cellular function, it is likely that they also have a unique pattern of gene expression, and a greater understanding of the functions of some of these genes may provide insight into DC biology. As these genetic data are generated, the corollary analyses on how different genes contribute to the function of DCs will also need to be conducted. We have been investigating some of the genes that are selectively expressed in DCs and have identified one that appears to play a significant role in limiting the lifespan of DCs, mitogen induced nuclear orphan receptor (MINOR), which will be the focus of this proposal. Our studies plan to investigate both the biology of this gene in DCs and determine whether its manipulation can be exploited for a therapeutic effect. The identification of this gene was made through a subtractive hybridization analysis between activated macrophages and DCs, which revealed a highly upregulated expression of the mouse homolog to human MINOR in mature DCs. Many studies have now focused on the features of DCs that allow them to be such potent APCs, including analyses of signals for activation that are important for DC function (such as co-stimulatory molecules, molecules involved in Ag processing and presentation, *etc.*). Once DCs have been activated, however, they are thought to have a relatively short lifespan *in vivo*, which presumably serves to limit clonal expansion in an immune response. Thus, it appears that DCs are messengers with a limited time to carry their Ag to secondary lymphoid organs and activate T cells in the context of a pro-inflammatory environment. While many apoptotic pathways are likely involved in the induction of cell death in DCs, MINOR is different in that its expression is both highly upregulated and relatively restricted to DCs and thus may provide an avenue that can be specifically manipulated in DCs. Also, it may provide some understanding regarding the mechanism of DC susceptibility to death. Thus we will in parallel investigate the biology of MINOR expression in DCs, the impact of inhibiting its expression, and how we can potentially manipulate its expression for therapeutic potential.

DC-based vaccines

One avenue of capitalizing on MINOR inhibition is through the potential for enhancement of DC vaccines. DC-based vaccines have been tested in a number of animal models, and have also been translated to clinical medicine in several trials. Thus, the hope for successful immunotherapy through this mechanism is high. To produce DC vaccines, there are two primary methods: First, precursor cells can be isolated, grown *ex vivo* and differentiated in culture, then subsequently re-infused; second they can be generated *in vivo* through the systemic administration of GM-CSF and/or Flt-3 ligand (FLT3L). While these approaches have led to some degree of tumor immunity, they have also had limitations [3]. The identification of tumor specific and tumor-associated Ags has led to therapies such as vaccination with recombinant viruses or DCs modified to express Ags but these have also had limited effects.[4-6]

Therapeutic cancer vaccination depends on effective transfer of Ag to DCs and trafficking of the DCs to the secondary lymphoid organs. While many clinical trials have been initiated with *ex vivo* generated DCs, for the most part, no long term cures have been achieved. Interestingly, DCs are more resistant to some apoptotic pathways than other cells as a result of expression of molecules such as FLICE inhibitory protein (cFLIP) which can block caspase 8 activation and the subsequent apoptotic cascade[7]. Additionally, signaling by TRANCE, or

CD154 have been shown to prevent apoptosis in DC [8,9]In spite of these, they generally have a very short lifespan *in vivo*. In fact, animal models and clinical trials suggest that one major issue with *ex vivo* expansion and loading of DCs followed by re-injection is that relatively few DCs successfully traffic to spleen or lymph nodes [10] and those that do are rapidly cleared by host CTL[11]. In In addition, NK cells can kill DCs through TRAIL mediated apoptosis[12], and further studies using labeled DCs suggest that they are replaced every 3-4 days[13], implying a limited window during which T cells can encounter DCs presenting Ag. Thus, it seems possible that extending longevity of DCs would allow for their improved immunogenicity.[14] and understanding some of the genes expressed by DCs may help to elucidate mechanisms of DC biology, since many checkpoints limit the immune response naturally, some of which target DCs.

Analysis of genes selectively expressed in DCs

DCs have many known properties that allow them to be potent APCs, and not surprisingly, in gene analysis molecules such as CD86, MHCII, and CD40 are upregulated upon activation. In order to elucidate other genes that are important for DC function, a subtractive hybridization analysis was undertaken in which gene expression by activated DCs was compared to that of activated macrophages. This analysis identified a number of genes that were either up or down-regulated in mature DCs, [1,15] including one with homology to human MINOR. Our Prelim Results suggest that its forced expression induces apoptosis, and its inhibition inhibits natural apoptosis in DCs, indicating that MINOR was an important gene to investigate. Thus, it was selected as a potentially significant gene both for basic DC function in that it may help regulate DC lifespan, thereby limiting uncontrolled T cell activation and also serve as a target for improving DC-based therapies. The basic premise of conducting these genetic analyses is that new insight will be gained from identifying genes, and based on its observed functional effects on DCs, MINOR appears to be a good candidate for further evaluation of its roles.

MINOR

As described above, mouse MINOR was identified as a gene that is highly upregulated and selectively expressed in DCs. This gene belongs to the Nur77 family of orphan receptors that includes Nur77 and the rat homologue of MINOR, Nor 1, which is involved in activation induced cell death of T cells[16,17] and also caspase-independent cell death of macrophages.[18] (See Prelim Results, Fig 2). The role of Nur77 in T cell development has been fairly well documented. Interestingly, Nor-1, appears to be functionally redundant with Nur77 not only in DNA binding specificity and ability to transactivate from the NBRE promoter, but also in its ability to induce apoptosis in T cells. Furthermore, a dominant negative Nur77 gene fragment lacking the transactivation domain blocks the induction of apoptosis by both Nur77 and Nor-1. In addition, transgenic mice expressing the rat MINOR homologue, under the control of the *lck* promoter, exhibited a 15 fold reduction in their thymocyte number, clearly showing the impact of this pathway on T cells[16]. Our preliminary results show that Nur77 is not expressed in DCs, thus we are hypothesizing that the mouse MINOR substitutes for the signal to induce apoptosis, in a selective fashion.

The actual mechanism of cell death induction by these molecules is not as clear. Nur77 is a nuclear, DNA binding, transcriptional regulator, so the most likely mechanism is via transcription of downstream effectors [19]However, other evidence suggests that Nur77 can mediate its apoptotic affect in the mitochondria[20]. Using a GFP tagged Nur77 to follow its movement through cells, a translocation from the nucleus to mitochondria was observed with concomitant cytochrome c release in the presence of apoptosis inducing stimuli in these studies. The mitochondrial localization of GFP-Nur77 and subsequent cytochrome c release was constitutive if the nuclear localization signals and DNA binding domain of this protein. Overexpression of a Nur77 dominant negative blocked both the mitochondrial translocation of Nur77 and its induction of apoptosis[21]. Thus, while the significance of this pathway in induction of cell death appears to be clear, its specific mechanism is not, but studies to elucidate the signaling pathways and localization of MINOR are beyond the scope of the presently proposed investigations.

DC apoptosis and siRNA

The first set of studies we will conduct will focus on elucidating the role of MINOR in DCs and also determining the potential effects of inhibiting the process by which DCs undergo cell death. As a precedent for using this mechanism to enhance potentially therapeutic responses, previous studies have shown that manipulation of the bcl-2 pathway led to inhibition of apoptotic cell death of DCs. DCs were shown to downregulate the anti-apoptotic bcl-2 upon maturation, leading to their progression to cell death. Mice were generated that were transgenic for bcl-2, that had increased numbers of DCs. Further, DCs from these transgenic mice as vaccines generated an enhanced immune response.[22] Also, while tumors can induce apoptosis of DCs and transduction of *ex vivo* generated DCs with the anti-apoptotic Bcl-xl increased their resistance to apoptosis and improved tumor vaccine efficacy.[23] Thus, it may be possible to improve DC vaccines through manipulation of apoptotic pathways. To this end, we have developed an siRNA that specifically knocks down expression of the MINOR gene. We show that this inhibits apoptosis of DCs *in vitro* and one goal of this study is to determine whether we can use this inhibition of DC apoptosis to enhance tumor vaccines. We will analyze its effects on both *ex vivo* generated DC vaccines as well as *in vivo* generated DCs. Since MINOR expression appears to be selective for DCs, unlike other anti-apoptotic approaches, we hypothesize that the potential for oncogenesis may be lessened through this approach; this will need to be further tested however.

Tracking immune responses

In order to follow the immune responses generated by the DCs, we will continue to use an Ag that is well-defined, Influenza hemagglutinin (HA), along with well-characterized anti-HA TCR transgenic (Tg) mice, to explore basic immunological processes, relevant to tumor immunity. We use the HA Ag and the pair of MHC class I (K^d) **(termed clone 4)** and II ($I\text{-}E^d$) restricted **(termed 6.5),** HA specific TCR Tg mice to track immune responses. In addition, one important feature of HA for the tumor studies proposed is that HA behaves like a natural tumor Ag in a number of tumor models such as the A20 B cell lymphoma, in that moderate levels of HA expression do not alter the biology, immunogenicity, or *in vivo* growth characteristics of the tumor[24-26]. In addition, we have transgenic mice available that express HA as self, which will allow us to investigate activation of tolerant T cells[26]. Thus, while this Ag may have limitations, it provides a strong foundation on which to develop this system and will allow us to address some important fundamental questions of immune responses. That said, in order to expand the system for investigating therapeutic avenues, we have developed an A20 lymphoma that expresses the Epstein Barr Virus Ag, LMP2, and are thus will be incorporating a second, naturally occurring, Ag in a tumor for future studies.

Enhancement of *ex vivo* DC vaccines:

DC based therapies have been investigated for tumors for which the antigen is known, through antigen-specific activation, and also for tumors for which the antigen is not known, through whole tumor cell-based activation. The use of *ex vivo* DC vaccines has the potential advantage of Ag loading with multiple Ags, some of which are not identified through the use of whole tumor cell lysate as a means to pulse DCs with Ag. In fact, previous studies have shown that tumor ignorance by $CD8^+$ T cells can be reversed if they are exposed to antigen-pulsed DCs. [27] Thus, while the potential for impact on anti-tumor immunity by DCs is clear, the trafficking and survival of these DCs have been significant limiting factors in their use, thus an enhancement to their survival could provide a critical improvement to this therapy.

One factor still to be resolved in the use of whole tumor cells is the controversy over whether the method used to prepare the tumor cells or Ag for loading into DCs affects the efficacy of stimulation. [28] One study demonstrated that apoptotic tumor cells were more efficient than lysates [29], although another group found no variability in lysate vs apoptotic cells. [30] Thus, one potential advantage of gene-modifying DCs with Ag

genes is that it may bypass this issue in that no prior Ag preparation is necessary. Tumor specific and associated Ags are being identified at a rapid rate, many of which would be suitable for testing in either this approach or the *in vivo* approach described next. Many different tumor-specific and associated Ags have been identified for both hematologic and solid malignancies. Our present model is based on a hematologic malignancy, thus the current focus will be on those Ags. Tumor antigens for hematologic malignancies can be broadly divided into 5 categories: (1) unique Ags, e.g., AML/ETO in AML or idiotypic epitopes; (2) shared tumor Ags, e.g., MAGE in multiple myeloma; (3) overexpressed Ags, e.g, PR-3 in AML & CML; (4) mutated oncogenic proteins, e.g., p53; (5) viral associated Ags, e.g., EBV. Many of the Ags have already been targeted by protein-based approaches, with varying levels of success. To extrapolate to a gene-based expression of the Ag would be a reasonable step. Thus, availability of genes encoding specific tumor antigens should not be a limiting resource for the HSC transduction section of this project.

Enhancing *in vivo* DC vaccines generated from transduced hematopoietic stem cells

Another potential avenue for enhancing immunotherapeutic responses is by improving DC survival in a model we have recently developed involving generation of DCs *in vivo* from hematopoietic stem-progenitor cells (termed HSCs in this application) that have been transduced with a model tumor Ag prior to transplantation followed by differentiation into DCs *in vivo* via administration of systemic agents. This method provides for efficient expression of Ag by DCs *in vivo*. The introduction of genes encoding Ag into the HSCs combines both effective delivery of Ag and also the benefits of autologous BMT (autoBMT), which is an important treatment strategy for a number of hematologic malignancies. The success of autoBMT may be due in part to the generation of a lymphopenic environment in which it is easier to re-direct the immune system towards tumor antigens, as shown with vaccines administered post BMT, including DC based vaccines. [31] Thus, we will begin to investigate whether these features combined with prolonged DC survival will produce an even greater cure rate of tumor bearing mice. In our previously conducted studies, we have employed lentiviral transduction of HSC and shown that this process results in expression of encoded Ag in a large proportion of DCs from both lymph node and spleen. To bypass the expected induction of central tolerance from repopulation of the thymus with Ag expressing DCs, we also infused mature post-thymic lymphocytes, [donor lymphocyte infusions or (DLI)]. To activate DCs for maximal presentation of Ag to T cells, we treated mice with FLT3L, which we obtained through an MTA with Amgen, and an activating antibody against CD40. FLT3L generates large numbers of DCs *in vivo* [3,32,33] that stimulate γIFN production [34] and CD40 activation has been shown to enhance the protective effect of anti-tumor vaccines, possibly through inhibition of peripheral tolerance. [35,36] Studies from my lab group show that this tripartite approach of HSC transduction, DLI and systemic DC activation leads to dramatic expansion of Ag specific T cells and successful treatment of established tumor in mice.[37] Despite a significant cure rate, some animals ultimately relapsed. Thus, the system would benefit from novel improvements, and one hypothesis to be tested in this application is that the addition of siRNA for MINOR will allow the DCs to be more resistant to killing, thus enhancing their efficacy.

In our model, activation of DCs was absolutely necessary to achieve a significant percentage of tumor regressors. However, our hypothesis is that activation of DCs is a double edged sword in that while it is necessary for maximal immune stimulation, that it also leads to an induction of MINOR, thereby rapidly initiating cell death. Thus, if we inhibit MINOR along with administration of activational agents it is possible that we can increase the immunogenic effect of DCs. By determining first whether there are differences in induction of MINOR by some of these candidate activators, we will have a rationale for determining which agents are most likely to produce an enhanced effect from the inhibition of MINOR. In order to optimize the system further, we will investigate alternatives for DC activators as well, since the number of available DC activational agents is increasing, and appear to have selectivity among different subsets of DCs in some cases. In addition, these agents are likely to lead to maturation of DCs through different pathways, some of which have not yet been elucidated. Thus, our studies will also investigate some of these different agents for both their overall effects on tumor regression in this model, and whether there is a correlation between their *in vivo* effects

and any observed differential effects on induction of MINOR. Our Prelim Results show that IL-4 and TNFα upregulate MINOR, and we will further assess other activators as well.

We have previously utilized an activating antibody against CD40 (αCD40), in our animal models, which is potent DC activator. Its activation of DCs may be important as well for its capacity to contribute to the prevention and/or reversal of tolerance, since interactions through CD154-CD40 have been shown to be involved in priming of CTL mediated by $CD4^+$ T cells[38]. This feature may be critical for tumor therapies, where it will be critical to activate T cells that may already have been exposed to tolerizing conditions. Previous studies have shown that tumor ignorance by $CD8^+$ T cells can be reversed if they are exposed to Ag-pulsed DCs [27], thus it is possible that if DCs can be kept alive long enough to activate T cells in the right setting, that their stimulatory capacity in these tolerizing settings can be greatly enhanced. Other molecules that activate DCs, primarily immunostimulatory synthetic CpG oligonucleotides have shown interesting effects in the context of stimulating tumor immunity. These CpGs act through toll like receptor (TLR)9 to activate both mature and immature DCs to upregulate co-stimulatory molecules *in vitro* [39,40]and *in vivo* to produce γIFN[41]. CpG oligos also inhibit apoptosis of DCs [42], which may contribute to their observed enhancement of DC vaccines[43]. Thus, the CpG oligo may substitute for the αCD40 or provide an additional stimulus. In addition to these DC stimulators, some more recently described agents stimulate DCs through TLR7 [44]and appear to produce a potent effect, possibly working through the selective stimulation of plasmacytoid DCs[45] (some are commercially available; in addition we have obtained one from 3M, reported above). While investigating mechanisms and functions of individual genes is necessary for understanding each one's contribution to DC biology, combination strategies may have the greatest potential ultimately to generate curative therapies. Thus, we will test these agents along with LPS, which has served as a prototypical DC activator to assess a mechanistic rationale for incorporating different DC activating agents in our therapeutic system.

In addition, the studies using siRNA-MINOR or siRNA-MINOR-HA transduced HSCs in non-tumor bearing mice will allow us to follow the course of DCs that are derived from transduced HSCs to determine how their fate and function differ from non-transduced DCs. The comparison of these two courses will allow us to determine some of the effects of MINOR expression on different subtypes of DCs and trafficking. It is possible that different subtypes will have differential susceptibility to MINOR-induced cell death and that the balance among these subtypes can be changed through inhibiting this gene. We may also find that immune responses in different organs are differentially changed, which we can assess through following HA-specific responses. For example, our (unpublished) and others' observations have been that plasmacytoid DCs are more susceptible to apoptosis than myeloid DCs[46]. Thus, while one specific goal will be to determine whether these two populations have differential expression of MINOR, parallel studies can investigate whether in vivo expression of siRNA -MINOR also produces a concomitant change in T cell responses in different organs, either by intensity of T cell expansion or the nature of the T cell response. These studies will provide evidence of a physiologic role of MINOR in *de novo* generated DCs in an otherwise unmanipulated environment.

C. PRELIMINARY RESULTS

Fig 1. MINOR expression is highly and selectively upregulated in mature DCs.

Initial studies utilized a subtractive hybridization strategy to identify genes that were selectively upregulated in mature DCs relative to activated macrophages. More extensive methodology and the function of one upregulated gene (B7-DC) have already been described in detail[1], but the goal of the subtractive hybridization study was to identify new genes that were specifically upregulated in mature DCs compared to less potent APCs (macrophages) and thus might contribute to the unique function of these cells. Of the genes that were in the screen, the mouse homolog of MINOR was one of the most highly and selectively upregulated gene in bone marrow-derived DCs. Both the virtual Northern analysis and quantitative PCR analysis showed dramatic upregulation of MINOR expression as shown below.

Fig 1a.

For the subtractive hybridization and all other studies to be described below, generation of BM-DCs was conducted by the same standard methods (shown in legend).

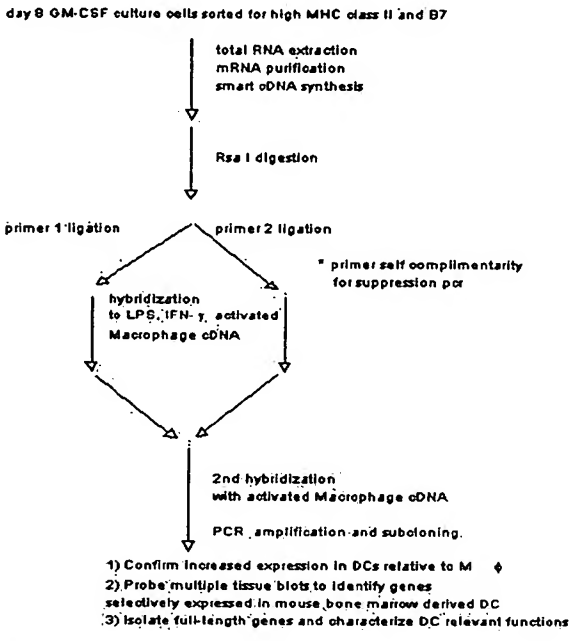


Fig 1a. Subtractive hybridization method.

The basic strategy for identifying genes particular to DCs is shown graphically in the flow chart to the left and previously published [1]. For all experiments described in the proposal, the basic procedure for generation of DCs is as follows: bone marrow cells are flushed from the femurs and tibias of mice, washed and cultured in 100-mm dishes (1 x 10^6 cells/ml) in 15 ml of complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg of streptomycin, 50 mM 2-ME, and 5% FCS (all from Life Technologies) supplemented with recombinant mouse GM-CSF (1000 U/ml; R&D). Nonadherent cells are removed on day 2 and 4 and replaced with fresh medium Depending on the experiment, either additional maturation agents or viral transduction is then conducted, as specified for each individual study. The initial screen identified 114 clones specifically upregulated in DCs, which were then further screened for redundancy and known function. Searches for homology to known genes revealed a number of interesting candidate genes for analysis.

Fig1b. Virtual Northern analysis

In order to confirm selective expression of genes that were identified by the subtractive hybridization in DCs, multiple tissues (labeled on top of blot) from different origins were analyzed for comparative expression of the new clones and also some with known function (eg, CD80 and CCR7). As the blot shows, MINOR expression was quite selective for DCs.

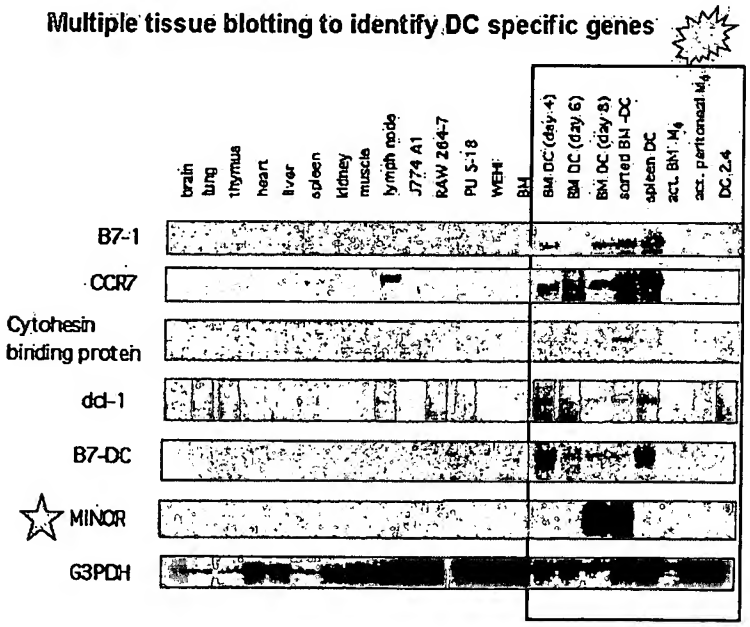


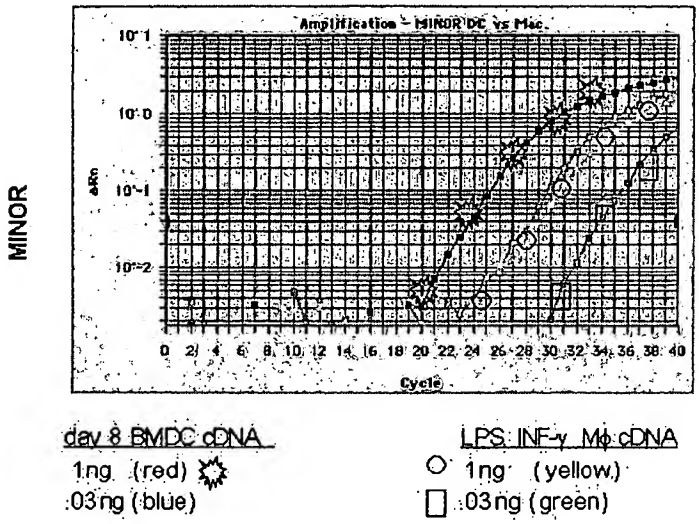
Fig 1b. Virtual Northern analysis of selectively expressed genes.

After more extensive analysis of the sequences that were upregulated in DCs, the number of candidate cDNA clones to further investigate was narrowed to 36. Shown left is the virtual Northern analysis of cDNAs from these different tissues probed for expression of some of the genes of interest. The box highlights DC and macrophage lineages, and expression of MINOR is marked with the star.

Fig 1c. Confirmation of expression by quantitative PCR analysis.

To confirm the relative expression levels of MINOR between DCs and activated macrophages, a qPCR analysis was developed and conducted. The top curve shows expression of MINOR for DCs and macrophages at 2 different RNA inputs, as depicted. The bottom graph shows the expression for 18s rRNA for the 2 cell groups at 2 different RNA inputs. As the figures below show, while the control 18s for DCs and macrophages are similar, MINOR is significantly upregulated for DCs.

MINOR qPCR



control qPCR for 18s rRNA:

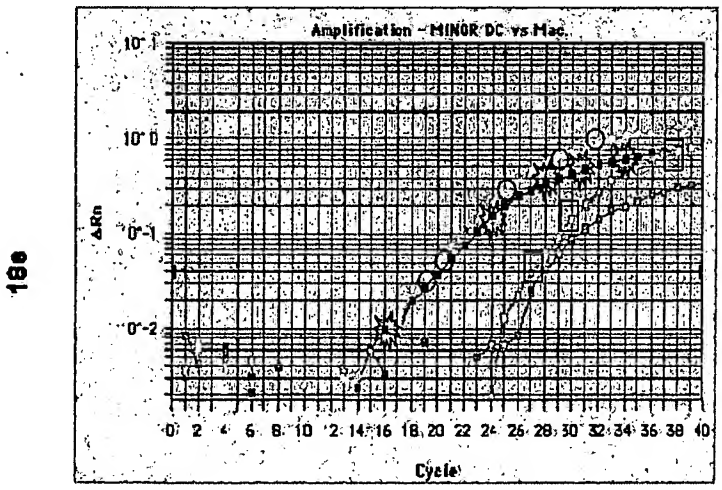
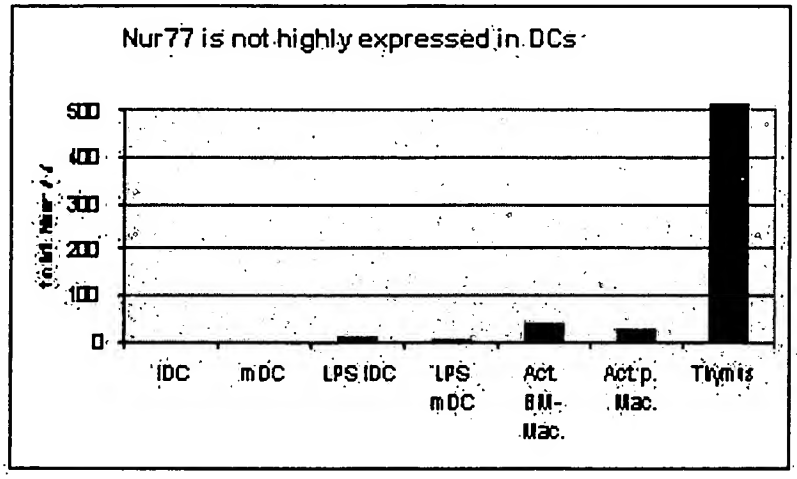


Fig 1c. qPCR analysis of MINOR.

After differentiation into either macrophages or DCs, cells were sorted for purity, total RNA extracted with TRIZOL, and cDNA synthesis conducted with an Invitrogen 1st strand synthesis kit. 1:9 cDNA dilutions were analyzed in duplicate. Standard curves were generated on the cycle threshold (Ct) vs log ng input RNA; then all samples were calculated with n=(Ct-b)/m. For calculating fold expression of MINOR, inverse logs were calculated and experimental values divided by 18s internal controls. Applied Biosystems PDAR reagents were used for detection of 18s rRNA and MINOR with universal Taqman Mastermix, with primers at 600nM and probes at 200nm. PCR was done using specific primer sets for each clone. 6FAM/TAMRA labeled probe CCCTTGCAGCCCTCGCAGGTG was used with flanking oligos TGCCAGCACTACGGAGTCC and TTCTGCACCGTTCTCTTGAAGA for specific detection of MINOR. Since the original curves are shown in color, shapes have been added to the lines and legends to clarify identity.

Fig 1d. Nur77 is not expressed by DCs; MINOR is expressed at high levels and can be induced to higher levels upon stimulation:

Since MINOR is a member of the Nur77 family, we wanted to determine whether Nur77 was also expressed, to assess redundancy of these genes. As expected, expression of Nur77 was high in thymus, consistent with its role in T cell apoptosis. However in DCs, expression of Nur77 was very low to undetectable, even in the activated DCs. In contrast, in unstimulated mature DCs, MINOR expression is relatively high and could be induced to even higher levels by both IL-4 and TNF-α.



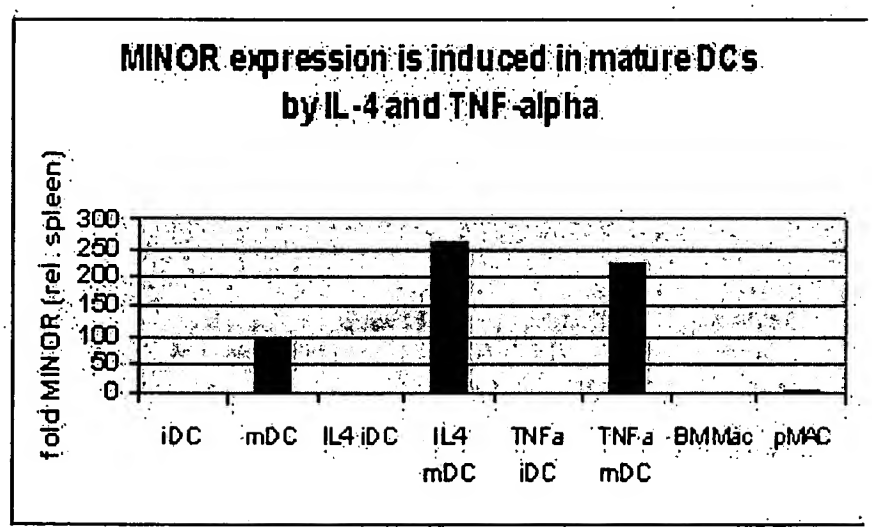
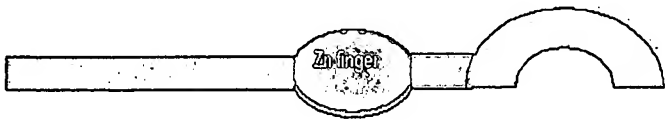


Fig 1d. Comparative expression of Nur77 and MINOR in DCs.

qPCR analysis was conducted as above to determine expression of Nur77 by DCs. Preparation of DCs, macrophages, and qPCR analysis was all as above. Nur77 qPCR was conducted essentially as described above, with primer sequences: TGATGTTCCCGCCTTTGC and GCAAAGGCGGGAACATCA. For stimulation, LPS (25 ng/ml), IL-4 (500u/ml), or TNF-α (500u/ml) was added to day 8 cultures for 24-48 hrs prior to harvest of cells for RNA isolation.

Fig 2. MINOR is a member of the Nur77 steroid receptor family

MINOR is a 627aa protein composed of a N-terminal transcriptional transactivating domain, a central zinc finger DNA binding domain with nuclear localization signals (aa290-361), and C-terminal steroid ligand binding domain (aa440-595).



subfamily	member	Nur77	Nurr1	MINOR
alpha	Nur77		35.3	45.7
beta	Nurr1	35.3		35.9
gamma	MINOR	45.7	35.9	

Fig 2. MINOR is a member of the Nur77 family.
A cartoon structure of MINOR and the results of protein sequence alignment of MINOR with other members of the Nur77/Nurr1 steroid hormone receptor family were performed and % identity to the most similar sequences are shown. The subfamilies of this gene are shown in the chart.

Fig 3. Overexpression of the MINOR gene induces apoptosis in a DC-like cell line.

In order to determine whether forced expression of the MINOR gene would induce apoptosis in a DC-like cell line, the DC 2.4 cell line, which is phenotypically similar to immature DCs [47] and does not express high levels of MINOR naturally, was gene modified with either a control GFP or Nor-1 GFP vector as described in the legend, then and analyzed for early (Annexin V+/AAD-); lower right quadrant on the FACS plot below, and late (Annexin V+/AAD+); upper right quadrant on plot, stages of cell death. As the figure shows, there was a significant increase in both Annexin V and 7-AAD positive cells if they constitutively expressed the Nor1 homolog of MINOR, suggesting that constitutive MINOR expression induces cell death. In all studies of apoptosis, great care is taken in controls and compensation to minimize artifacts due to autofluorescence of dying cells.

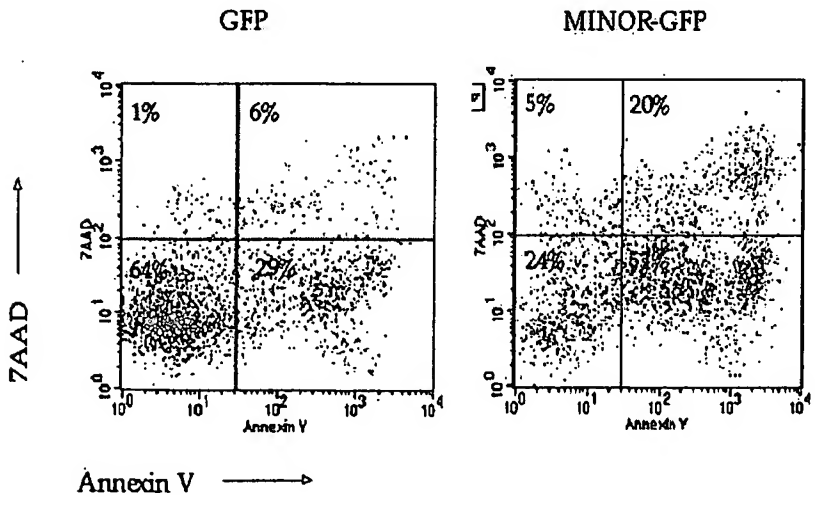


Fig 3. The MINOR homolog Nor1 induces apoptosis in DC2.4 cells.

The DC-like cell line DC 2.4 was transfected with either the rat homolog MINOR cDNA, {pCI Nor-1, which was obtained from Astar Winoto and inserted into HindIII-BamHI digested peGFPN2 (Clonetech)} or pEGFP-N2 was used as a GFP alone control. Expression of Nor-1 in these cells was achieved by plasmid DNA transfection using Lipofectamine 2000 reagent as directed (GIBCO-BRL). 2-4 days later, cells were harvested and stained with Annexin V/ 7-AAD using the manufacturer's recommended protocol, and analyzed by FACS for cell death. For the graphs shown, cells were gated on GFP+ populations in order to specifically compare transduced cells.

Fig 4. Transduction with siRNA for MINOR shows a potent knockdown of MINOR expression.

In order to validate that the siRNA was functionally decreasing the expression of MINOR, a quantitative PCR analysis was conducted with MINOR specific primers. As the graph shows, while unmodified mature DCs express a high level of MINOR, transduction with siRNA-MINOR led to a 90% knockdown in expression .

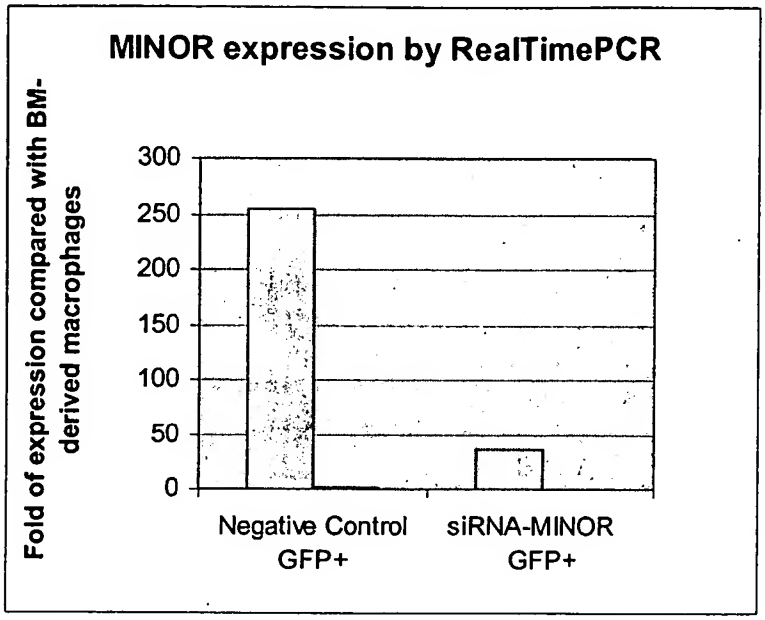


Fig 4. Generation of a lentivirus-based vector expressing siRNA to genetically suppress MINOR expression

Since the siRNA construct has not yet been published, the following is a complete description of the methodology: To generate a vector-based suppression of MINOR expression, the construct pSUPER-retro (Oligoengine) was employed as a template. The siRNA oligonucleotides designed contained a sense strand of 19 nucleotide sequence followed by a short spacer (TTCAAGAGA), the reverse complement of the sense strand, and five thymidines as a RNA pol III transcriptional stop signal. Briefly, the pSUPER-retro vector was digested with BglII and HindIII and the annealed oligos (5'-GATCCCCTGCCCT TGTCCGAGCTTTATTCAAGAGATAAAGCTCGGACAAGGGCATTTTTGGAAA-3'; forward and 5'AGCTTTTCCAAA AATGCCCTTGTCCGAGCTTTATCTCTTGAATAAAGCTCGGACAAGGGCAGGG-3'; reverse) were ligated into the vector according to the manufacturer's protocol. To construct lentivectors encoding our siRNA construct, the complete human H1-RNA promoter and the siRNA cassette and the PGK promoter were subcloned at XhoI and NheI 5' of the reporter eGFP gene of the third generation self-inactivating lentiviral vector, Sin-18 provided by D. Trono.[2] All inserts were sequenced.

A 3-plasmid transfection system was employed to generate high-titer lentivirus as previously described (Cui et al., 2003). Briefly, 293 T cells were grown to 80% confluency on a 100-mm cell culture dish and transfected with 10 μg of pCMV-8.9, 2.5 μg of pMD.G, 5 μg of LV-siRNA using the Lipofectamine 2000 (Life Technologies). Supernatants containing the virus were collected at 24 and 48 hour post-transfection, pooled and filtered through a 0.2-μm filter. The titer of the virus, measured in transducing units (TU), was determined using 293 T cells and analyzed by FACS analysis (by GFP).

DC were generated from bone marrow progenitor cells,as described above, transduced on days 3-6 of culture, harvested on day 8, and evaluated by FACS for phenotype and RNA for quantitative PCR.

Fig 5. Transduction with siRNA for MINOR inhibited apoptosis in BM-derived DCs in primary culture.

To determine whether the siRNA-MINOR would inhibit cell death in primary BM-derived cultures, BM-DCs were generated, transduced, and analyzed for natural apoptosis. The GFP^- fractions in both populations represent the untransduced groups, while the GFP^+ populations are transduced. The control GFP^+ populations express a control siRNA-GFP while the siRNA-GFP population expresses both GFP and siRNA-MINOR. Thus, the effects of GFP (if any) are controlled for. As the plots show, while there is no significant difference in the GFP- fractions from either group or within the GFP+ fraction in the control, the GFP+ fraction transduced with siRNA for MINOR had a significant decrease in cell death, demonstrating that transduction with the siRNA can decrease DC apoptosis.

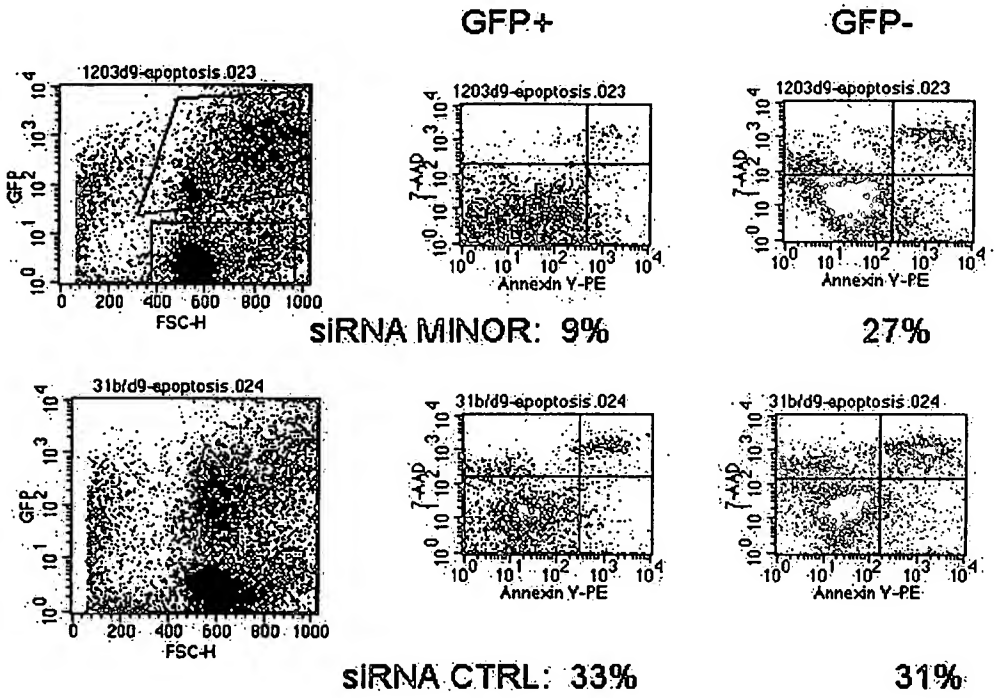


Fig 5. Inhibition of apoptosis by siRNA-MINOR.

Bone marrow DCs were generated *in vitro*, (as described but briefly, bone marrow cells were flushed from the femurs and tibias of Balb/c mice, washed and cultured in complete medium (RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg of streptomycin, 50 mM 2-ME, and 5% FCS (all purchased from Life Technologies) supplemented with recombinant mouse GM-CSF (1000 U/ml; R&D). All cultures were incubated at 37 °C in 5% humidified CO2. Nonadherent cells were removed on day 2 and 4 and replaced with fresh medium. Cells were subjected to three rounds of lentiviral transduction with concentrated virus (i.e. MOI = 5) on day 2, 4 and 6 in the presence of 8 μg/ml polybrene. After 6 days of cultures, the cells in suspension were reseeded in complete medium with and on days 5 & 6 were transduced with the LV encoding the siRNA-MINOR-GFP or control siRNA-GFP (sequence gtatacgtgtttgctcccctt, no known homology to any gene). On day 9, cells were analyzed for induction of cell death. Shown are FACS plots gated either on $GFP^{negative}$ (right) or GFP+ (left) for either MINOR siRNA (left) or control (right) for Annexin V vs 7-AAD (indicating the dead populations). Perentages of dead cells are calculated from both right and the upper left quandrants all together. As the plots show, there is no significant difference in cell death in the $GFP^{-negative}$ fractions in both populations or the GFP+ in the control. In contrast, there is a significant decrease in cell death in the transduced (GFP^+) fraction in the siRNA-MINOR expressing population, indicating an inhibition of apoptosis. (1 of 3 representative expts shown).

Figure 6. Transduction of *ex vivo* DCs with siRNA-MINOR enhances their vaccine potency.

Since it appeared that transduction with siRNA-MINOR could inhibit cell death, we hypothesized that it would also enhance the immunogenicity of BM-DCs when used as a vaccine. As the graph shows, there was a significant enhancement in stimulation of Ag-specific T cells, if the DCs expressed siRNA-MINOR along with Ag (HA in this case). In these first studies, DCs were not sorted prior to infusion, thus only a fraction (20-30%) of the infused DCs actually expressed the gene. Future experiments will determine the effect of sorted DCs.

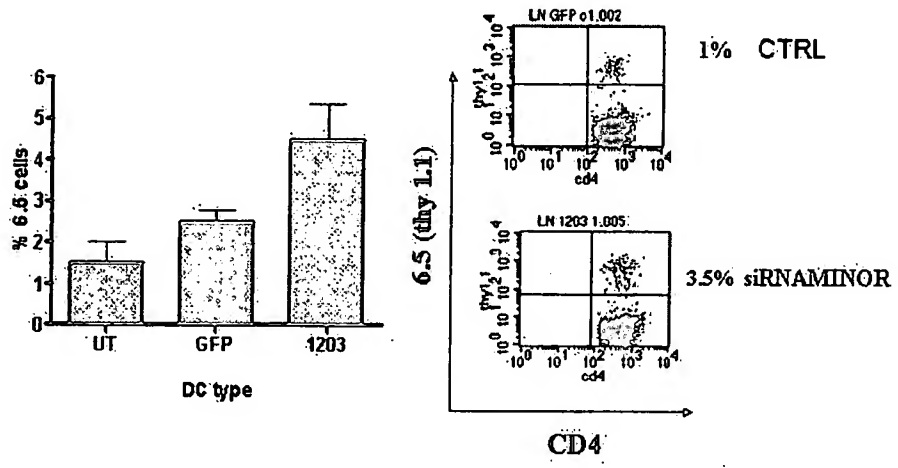


Fig 6. siRNA-MINOR transduction of *ex vivo* generated, HA pulsed DCs enhances their immunogenicity.

For these studies, DCs were generated from BM, transduced with either GFP or siRNA-MINOR-GFP and pulsed both groups with the class II restricted peptide for HA. To track the T cell responses, we adoptively transferred 6.5 T cells (here on a thy1.1 background), and 5 days later sacrificed the mice to determine T cell expansion in spleen and lymph nodes. Shown are a representative FACS plot, stained for CD4 and thy 1.1 form the siRNA=MINOR (left, and control siRNA-GFP (right). Below is the average and SD for all mice (2 expts, 6 mice / vector).

The basic methodology employed in Figs 7-9 is the same and will describe the *in vivo* generation of DCs from transduced HSCs used for BMT. For all these figures, the following experimental procedure was conducted: BALB/c BM was harvested and enriched for HSCs using the StemSep separation kit (Stem Cell Technologies). HSCs were transduced for 3 days with either the control siRNA-GFP LV or the siRNA-MINOR GFP and then transplanted into myeloablatively (850cGy radiated) treated BALB/c mice. After 8-10 weeks of engraftment time, mice were sacrificed and their spleens and lymph nodes harvested for DC isolation. Figures 7-9 show representative FACS plots and total averages and statistics for all mice in the groups:

Figure 7. Transduction of HSC with MINOR siRNA confers selective protection to DC populations.

In order to assess relative expression of the siRNA-MINOR in CD11c+ cells, compared to control siRNA expression, FACS analysis was used to compare GFP by CD11c. If no selective expression were present, then the ratio of GFP in CD11c+ to CD11c- should be equal. However, a comparison of the percentage of CD11c+ cells that contained the vector with the percentage of CD11c- cells that contained vector showed that the there was preferential vector expression of siRNA-MINOR in CD11c+ relative to CD11c- compared to control GFP+ in CD11c+ relative to CD11c-. In other words, the ratio of GFP is the same in the CD11c pos and neg fractions in the control, but was shifted towards CD11c pos cells in the siRNA MINOR, consistent with the notion that siRNA-MINOR confers a survival advantage to DCs, thereby preserving the CD11c population.

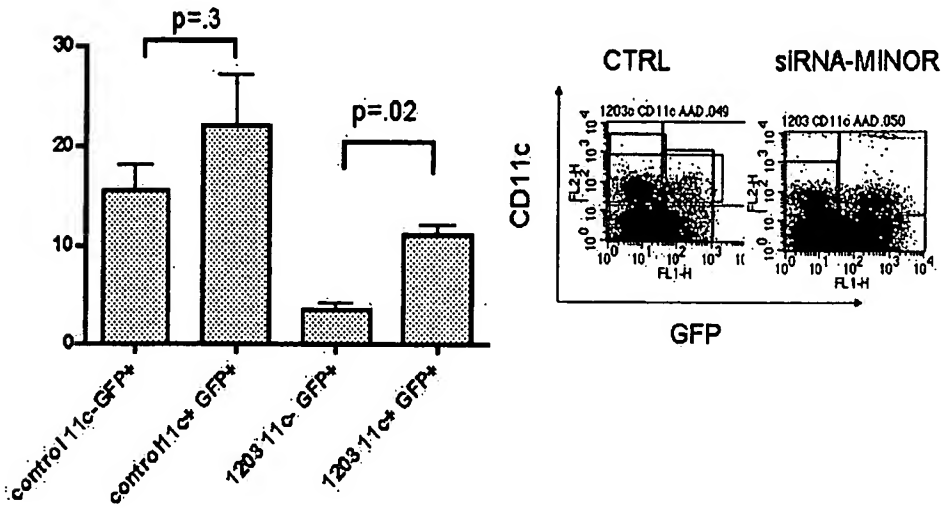


Fig. 7. Expression of siRNA-MINOR in CD11c+ cells. Following harvest, spleens and LN were stained for CD11c and 7-AAD. Shown are representative plots of LN for GFP by CD11c, in order to compare relative expression of the vectors in DC (upper 2 quads) vs non-DC (lower 2 quads) populations. The graph shows the average and SD of 6 mice/group. The statistics show that while there is no significant difference in the GFP expression by CD11c+ and CD11c- cells in the control group (labeled 1203c on the FACS), that there is a significant difference in the siRNA-MINOR group (labeled 1203).

Fig 8.. siRNA MINOR is preferentially maintained in the CD86^{hi} populations.

A similar analysis was conducted for expression of the vectors based on CD86 expression, in order to assess levels of expression as related to DC maturity. The ratios of GFP+ cells in the CD86^{hi} and CD86$^{lo\text{-}intmdt}$ were compared. The ratios of upper left to lower left should be the same as upper right to lower right, if no selective expression were involved. As the comparisons show, however, while in the GFP control, there is no significant difference in these ratios, in the siRNA-MINOR, there is a much higher percentage of GFP+ cells in the CD86^{hi} group, indicating that this group is the most selectively affected.

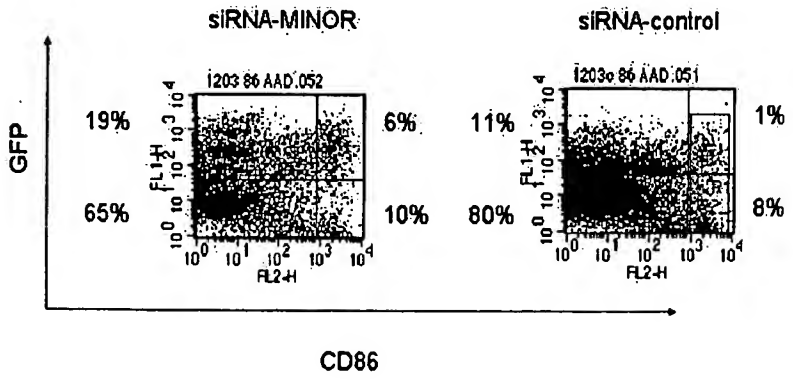


Fig 8. siRNA-MINOR is selectively expressed in CD86^{hi} cells.
As in the previous figure, LN cells were enriched for DCs, and then stained with CD86 and 7-AAD. Shown are representative FACS plots for siRNA-MINOR, left, and the control, right, for CD86 by GFP.

Figure 9. siRNA-MINOR increases the viability of DC progeny of transduced HSCs

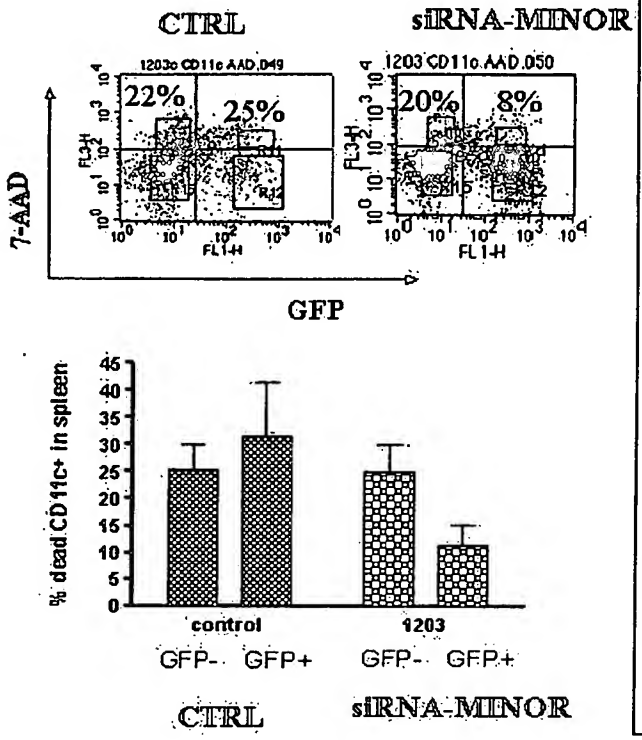


Fig 9. siRNA transduction of HSCs prior to transplant results in a decrease in DC death.
Following reconstitution, mice were analyzed for the percentage of $CD11c^+$ cells that were alive. Two separate comparisons were made: One within each group of mice (control GFP^+ vs control GFP^- and siRNA-MINOR GFP^+ vs siRNA-MINOR GFP^+ As the representative FACS plots for the internal control show, and the averages of each in the graph, there is a significant difference between the MINORsiRNA GFP^+ and GFP^- and the GFP^+ vs control GFP^+, indicating a selective protection from the siRNA-MINOR. In order to more accurately analyze the FACS plots, 2 separate statistics were generated. First is the standard quad stats, and second is a more confined region stat analysis, in order to eliminate the upper right cells that fall on the 45^0 line. While the differences are actually more dramatic using the quad stats, we believe that it is more precise to use the region stats (percentages shown in the plots and graphs) in which we can more reliably quantify true GFP signal from autofluorescence. Future experiments will include other means of distinguishing transduced cells to avoid this issue.

Fig 10. Tumor therapy system using transduced HSCs for BMT.

One goal of the current proposal is to build on a model system we have been developing. We have previously reported that transduction of HSCs with HA as a tumor Ag and treatment with FLT3L and αCD40 results in a cure in a significant number of tumor bearing mice in which the tumor expresses HA. The data suggest that activation of DCs and provision of $CD8^+$ T cells are necessary for cure. However, not all animals were cured, and one of the goals of this proposal is to utilize the protection of DCs from killing by inhibiting MINOR to attempt to improve this therapy. One specific question in these studies is whether MINOR inhibition will lead to enhanced help for the added CD8s through better stimulation. The original model system was established by injecting A20-HA (previously described, a B cell lymphoma expressing HA as a model tumor Ag [50]), followed by BMT with gene modified HSCs for transplant (modified with either the control nerve growth factor receptor (NGFR) or HA. Following systemic administration of FLT3L, αCD40, and donor lymphocytes, a significant percentage of mice were cured if 3 components were present: HA in the HSCs, FLT3L/αCD40 and $CD8^+$ T cells Figure 10 shows the schema employed, with the following groups:

(1): NGFR transduced HSC + whole spleen DLI

(2) HA transduced HSC + whole spleen DLI

(3) HA transduced HSC + DLI depleted of CD4 cells

(4) HA transduced HSC + DLI depleted of CD8 cells

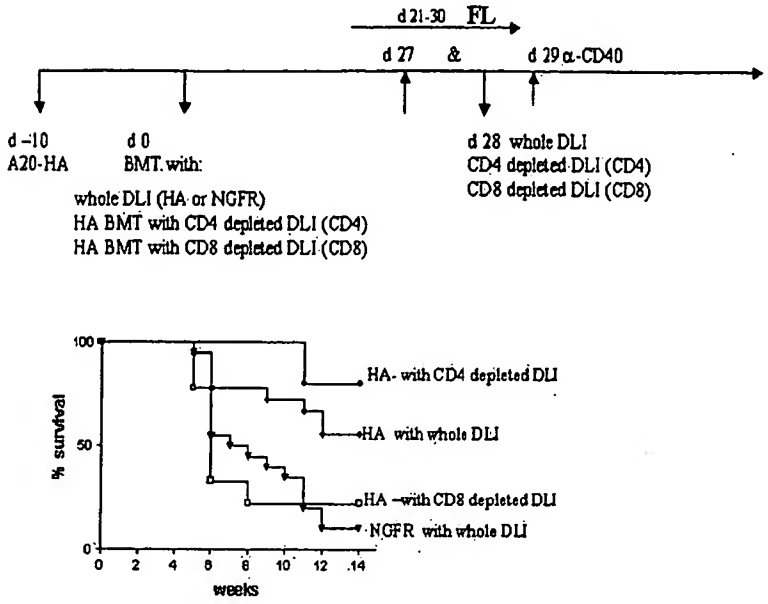


Figure 10. BMT with Ag transduced HSCs leads to a CD8+ dependent regression of tumors. In this study, mice were inoculated with A20-HA, and transplanted as before, with the exception being that the spleen cells given as DLI were depleted of either $CD4^+$ or $CD8^+$ T cells via MACS bead separation. 2.5 x 10^7 cells were administered both at the time of transplant and concurrently with the FL, as shown in the schematic. Mice were then followed for survival.

As figure 10 shows, the anti-tumor effect was lost in the absence of $CD8^+$ cells, ($p= 0.03$ between whole DLI and CD8 depleted DLI) demonstrating that these were absolutely necessary. $CD4^+$ depletion did not result in a negative effect, and even had a slight trend towards enhancing the effect, although this did not reach statistical significance.

As a technical aside, in our results we have tested both control siRNA-GFP or GFP alone as control vectors, with no detectable differences. For most experiments, we will continue to use the control siRNA-GFP to most accurately control for transduction; however we believe that the two are interchangeable. Similarly, we have used pulsing of cells with HA peptide as an alternative to transduction of cells with HA lentivirus and found equivalent presentation for *in vitro* studies; thus we may use either the dual construct vector with HA-siRNA MINOR or single siRNA-MINOR with pulsing with HA depending on the specific experimental design. Whenever possible, we have also used both Annexin V and 7-AAD together to more precisely quantify stages of apoptosis; however as necessary, only one will be used in order to reserve the channels for more accurate DC marker phenotyping.

D. EXPERIMENTAL DESIGN

The plan for the experimental design is to systematically evaluate the effects of MINOR expression and inhibition on DC function to evaluate its role in DCs, and in parallel to determine whether by manipulating its expression, we can improve immune responses and current approaches to cancer vaccines. Thus the aims will incorporate both investigations of DC biology and also applications to therapeutic models.

Aim 1. Evaluate the physiologic role of MINOR in DC longevity and function.

Aim 1a. Determine the *in vivo* expression of MINOR in different subsets of DCs before and after stimulation.

To further investigate the biology of MINOR expression, we will first compare levels in DC subsets from both unmanipulated mice and also mice exposed to varying DC activation agents. For these studies, we will isolate DCs from mice exposed to the following conditions: (1) naïve, (2) αCD40 (3) CpG oligos (4) a

All publications, patents and patent applications disclosed herein are incorporated into this application by reference in their entirety.

For example: "Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (volumes I-III) 1989, Cold Spring Harbor Laboratory Press, USA", "Harlowe and Lane, Antibodies a Laboratory Manual 1988 and 1998, Cold Spring Harbor Laboratory Press, USA" and "Ausubel *et al.*, Current Protocols 2001, John Wiley and sons, Inc." provide sections describing methodology for antibody generation and purification, diagnostic platforms, cloning procedures, etc. that may be used in the practice of the instant invention.

2. Problem Solved - continued:

Claims:

1. A new dendritic cell-selective gene, MINOR, capable of inducing apoptosis in dendritic cells
2. Manipulation of MINOR to develop vaccines for cancer through ex vivo DC-based vaccines using molecular targeting, e.g, siRNA.
3. Manipulation of MINOR to develop vaccines for cancer through ex vivo DC-based vaccines using molecular targeting, e.g, signal transduction inhibitors.
4. Manipulation of MINOR to develop vaccines for cancer through ex vivo DC-based vaccines using cell based targeting, e.g, cellular delivery of MINOR targeting molecules.
5. The use of MINOR reagents to classify and/or isolate specific populations of DCs.
6. Manipulation of MINOR to develop vaccines for cancer through blood or marrow transplantation with gene modified cells for transplant, by means of introduction of MINOR inhibiting sequences or genes.
7. Manipulation of MINOR to develop vaccines for cancer through blood or marrow transplantation with cells for transplant treated with MINOR-targeted signal transduction inhibitors or by using cell-based inhibition for delivery to the stem-progenitor cells used for transplant.
8. Manipulation of MINOR by molecular targeting, e.g, siRNA, to develop antigen specific immunity
9. Manipulation of MINOR by molecular targeting, e.g, signal transduction inhibitors, to develop antigen specific immunity.
10. Manipulation of MINOR by cell based targeting, e.g, cellular delivery of MINOR targeting molecules.to develop antigen specific immunity.
11. Manipulation of MINOR to enhance general immunogenicity of cells by cell based targeting, e.g, cellular delivery of MINOR targeting molecules.
12. Manipulation of MINOR to enhance general immunogenicity of cells by gene based approaches for targeting MINOR in immune cells.
13. Manipulation of MINOR to develop vaccines for viral and or bacterial disease for therapy and prophylaxis.
14. Manipulation of MINOR to develop vaccines for immunodeficiencies of unknown origin through inhibition of MINOR to enhance immunogenicity, by siRNA, small molecular weight compounds, or cell based approaches.
15. Manipulation of MINOR in antigen presenting cells by gene or cell-based approaches, to develop novel adjuvants for vaccines for cancers and infectious disease.
16. Potentiation of MINOR as an immunosuppressant for autoimmune or hyperimmune syndromes or to induce tolerance